APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1987, p. 193–195
0099-2240/87/010193-03$02.00/0

Vol. 53, No. 1

Identification of Environmental *Vibrio vulnificus* Isolates with a DNA Probe for the Cytotoxin-Hemolysin Gene

J. GLENN MORRIS, JR.,[1]* ANITA C. WRIGHT,[1] DIANE M. ROBERTS,[2] PATRICK K. WOOD,[1] LINDA M. SIMPSON,[2] AND JAMES D. OLIVER[2]

Division of Geographic Medicine, Department of Medicine, University of Maryland School of Medicine, Baltimore, Maryland 21201,[1] and Department of Biology, University of North Carolina at Charlotte, Charlotte, North Carolina 28223[2]

Received 29 May 1986/Accepted 30 September 1986

We screened 44 lactose-positive *Vibrio* strains isolated from the marine environment for homology with a 3.2-kilobase DNA fragment encoding the *Vibrio vulnificus* cytotoxin-hemolysin gene. All 29 marine isolates identified as *V. vulnificus* on the basis of numerical taxonomy and DNA-DNA hybridization studies hybridized with the cytotoxin gene probe, as did all *V. vulnificus* reference strains. Homologous gene sequences were identified in no other lactose-positive marine vibrio isolates nor in 10 other *Vibrio* species.

Vibrio vulnificus is an estuarine bacterial species that has been implicated as a cause of serious human wound infections and a syndrome of primary septicemia (1, 9, 14). Over 50% of patients with primary septicemia die; susceptible persons are thought to acquire the organism by eating raw oysters (9). *V. vulnificus* was first characterized on the basis of lactose fermentation, with papers referring to the organism simply as a "lactose-positive marine vibrio" (1). Subsequent studies with numerical taxonomy and DNA-DNA hybridization techniques have shown that there is significant heterogeneity among lactose-positive environmental *Vibrio* isolates (12, 15). In one study, only 20% of such isolates were identified as *V. vulnificus*, with most of the remainder not readily classifiable into recognized *Vibrio* species (12). There is currently no way of rapidly differentiating *V. vulnificus* (with its potential for causing serious human disease) from other lactose-positive environmental *Vibrio* isolates.

V. vulnificus produces a cytotoxin-hemolysin that has been implicated as a virulence factor for the organism (2); while the cytotoxin may contribute to virulence, it does not appear to be a marker for pathogenicity, as it is produced by both virulent and avirulent strains (J. G. Morris, A. C. Wright, L. M. Simpson, P. K. Wood, D. E. Johnson, and J. D. Oliver, FEMS Microbiol. Lett., in press). We isolated the structural gene for the cytotoxin on a 3.2-kilobase *Eco*RI-*Hin*dIII fragment cloned into pBR325, forming the plasmid pCVD702. In previous experiments, this fragment was shown to hybridize with *V. vulnificus* isolates but not with isolates from a limited number of other bacterial species (18). In the current study, we used this fragment as a DNA probe to screen 44 lactose-positive vibrios isolated during a series of environmental studies conducted along the Atlantic coast (12).

These marine isolates, with 23 reference *Vibrio* strains, had been screened for the presence of 47 phenotypic traits and fell into five taxonomic clusters (12). All *V. vulnificus* reference strains were in cluster III. Ten randomly selected environmental strains from this cluster which were examined for genetic homology to *V. vulnificus* by DNA-DNA hybridization were found to be *V. vulnificus*, with homologies of 77 to 95% at stringent temperatures (12). For the present study, several isolates in each of the remaining four clusters were similarly examined for homology to a reference strain of *V. vulnificus*. All were ≤22% related, with an average of 11% homology.

Members of all five clusters, as well as additional previously untested reference strains, were screened for homology with the DNA fragment encoding the *V. vulnificus* cytotoxin-hemolysin gene. pCVD702 was digested with *Eco*RI and *Hin*dIII, and the 3.2-kilobase fragment was isolated and labeled with [α-^{32}P]dATP (New England Nuclear Corp., Boston, Mass.) by nick translation (7). Colony hybridizations were performed under high-stringency conditions, as described by Moseley et al. (10). The results are shown in Table 1. All *V. vulnificus* reference strains and all isolates in cluster III hybridized with the gene probe. No hybridization was seen with reference strains from other bacterial species or with isolates from the remaining four clusters of lactose-positive vibrios.

Southern blot analyses (13) of chromosomal restriction endonuclease digests with the cytotoxin-hemolysin gene probe were performed for 16 *V. vulnificus* reference strains and 5 isolates from cluster III. Three Southern blot patterns were identified after *Hin*dIII digestion (Fig. 1); 9 strains showed pattern A, 2 strains showed pattern B, and 10 strains (including all three biogroup 2 reference strains [15]) showed pattern C. Two patterns were seen after digestion with either *Bam*HI or *Eco*RI (data not shown). It was not possible to correlate specific Southern blot patterns with characteristics such as isolate source. In contrast to observations made with toxins and hemolysins produced by other *Vibrio* species (8, 11), we did not see clear evidence of duplication of the cytotoxin-hemolysin gene. The significance of the faint hybridization seen with a second chromosomal fragment remains to be determined.

As oysters appear to be the major source for human *V. vulnificus* infections, we screened spiked oyster homogenates to determine if the probe could be used to identify *V. vulnificus* in oysters. Fifty grams of shucked oyster meats (obtained from a local retail distributor) were homogenized in 450 ml of phosphate-buffered saline (17). Undiluted homogenate and 1:10 and 1:100 dilutions of the homogenate were seeded with serial 10-fold dilutions of *V. vulnificus*, with concentrations ranging from 10^7 to 10^{10} CFU/ml; counts were confirmed by plating on thiosulfate-citrate-bile salts-

* Corresponding author.

TABLE 1. Hybridization of lactose-positive marine vibrio isolates and reference strains with a DNA probe for the *V. vulnificus* cytotoxin-hemolysin gene

Marine vibrio or reference strain	No. of isolates hybridizing with probe/no. tested
Lactose-positive marine vibrios[a]	
Cluster	
I	0/7
II	0/4
III	29/29
IV	0/1
V	0/3
Reference strain	
V. vulnificus	
Biogroup 1, clinical isolates	13/13
Biogroup 1, environmental isolates	4/4
Biogroup 2[b]	3/3
V. harveyi	0/14
V. campbellii	0/4
V. aesturianus	0/2
V. mimicus	0/2
V. parahaemolyticus	0/2
V. alginolyticus	0/1
V. anguillarum	0/1
V. cholerae	0/1
V. fischeri	0/1
V. fluvialis	0/1
Escherichia coli	0/3
Pseudomonas aeruginosa	0/1
Yersinia enterocolitica	0/1
Aeromonas hydrophila	0/1
Photobacterium leiognathi	0/1

[a] Described in reference 12. Clusters: I, H_2S^+, many bioluminescent; II, H_2S^+; IV, H_2S variable, urease positive, colistin resistant; V, H_2S^+, urease variable, bioluminescent.

[b] Isolated from eels; see reference 16.

sucrose agar. Ten-microliter samples were spotted onto nitrocellulose filters. The filters were incubated overnight on L agar, processed, and hybridized with the DNA probe. Sixty *V. vulnificus* organisms (10 μl of homogenate containing 6×10^3 CFU/ml) were easily detectable with the probe, with six *V. vulnificus* organisms producing a visible pattern on the autoradiograph. The results were unaffected by the initial concentration (i.e., undiluted, 1:10, or 1:100) of the oyster homogenate.

Our work indicates that the cytotoxin-hemolysin gene probe provides a very sensitive and specific means of identifying environmental *V. vulnificus* isolates, giving results comparable to those obtained by using much more complex numerical taxonomy and DNA-DNA hybridization techniques. Southern blot patterns have been used as markers in epidemiologic studies of other *Vibrio* species (5). Although we found no immediate correlation between characteristics such as isolate source and Southern blot pattern, these profiles may be useful as epidemiologic markers in subsequent clinical and environmental studies. DNA probes have been used to screen food and stool samples directly for a variety of pathogenic microorganisms (3, 4, 6). We were able to detect as few as 6 CFU per 10-μl sample when crude oyster homogenates were directly spotted onto nitrocellulose filters; however, with a 10-μl sample, as described, we could not consistently detect $<10^4$ CFU/g of oyster meat.

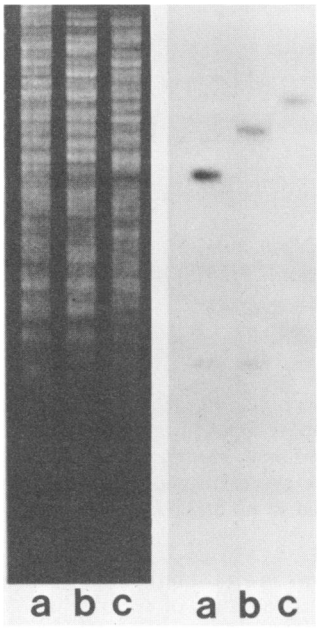

FIG. 1. *Hin*dIII digests of chromosomal DNA (left panel) and Southern blot hybridizations (right panel) with the 3.2-kilobase DNA fragment containing the *V. vulnificus* cytotoxin-hemolysin gene, with representative *V. vulnificus* strains. Lanes: a, *V. vulnificus* E4125; b, *V. vulnificus* EDL-174; c, *V. vulnificus* 9598 (biogroup 2).

While it will probably be necessary to use a larger sample volume or include concentration or enrichment steps to improve the sensitivity of the procedure, our data suggest that the cytotoxin-hemolysin gene probe is potentially a valuable tool for rapid, direct identification of *V. vulnificus* in environmental samples such as oysters or seawater.

This work was supported in part by Public Health Service grant 1RO1AI22189-O1A1 and a subcontract from Booz, Allen and Hamilton under Task XXVII, U.S. Food and Drug Administration contract 223-80-2295.

LITERATURE CITED

1. **Blake, P. A., M. H. Merson, R. E. Weaver, D. G. Hollis, and P. C. Heublein.** 1979. Disease caused by a marine Vibrio: clinical characteristics and epidemiology. N. Engl. J. Med. **300:** 1–5.
2. **Gray, L. D., and A. S. Kreger.** 1985. Purification and characterization of an extracellular cytolysin produced by *Vibrio vulnificus*. Infect. Immun. **48:**62–72.
3. **Hill, W. E.** 1981. DNA hybridization method for detecting enterotoxigenic *Escherichia coli* in human isolates and its possible application to food samples. J. Food Safety **3:**233–247.
4. **Hill, W. E., W. L. Payne, and C. C. G. Aulisio.** 1983. Detection and enumeration of virulent *Yersinia enterocolitica* in food by DNA colony hybridization. Appl. Environ. Microbiol. **46:** 636–641.
5. **Kaper, J. B., H. B. Bradford, N. C. Roberts, and S. Falkow.** 1982. Molecular epidemiology of *Vibrio cholerae* in the U.S. Gulf Coast. J. Clin. Microbiol. **16:**129–134.
6. **Lanata, C. F., J. B. Kaper, M. M. Baldini, R. E. Black, and M. M. Levine.** 1985. Sensitivity and specificity of DNA probes with the stool blot technique for detection of *Escherichia coli* enterotoxins. J. Infect. Dis. **152:**1087–1090.
7. **Maniatis, T., A. Jeffry, and A. G. Kleid.** 1975. Nucleotide sequence of the rightward operator of phage lambda. Proc. Natl.

Acad. Sci. USA **72**:1184–1188.
8. **Mekalanos, J. J.** 1983. Duplication and amplification of toxin genes in *Vibrio cholerae*. Cell **35**:253–263.
9. **Morris, J. G., Jr., and R. E. Black.** 1985. Cholera and other vibrioses in the United States. N. Engl. J. Med. **312**:343–350.
10. **Moseley, S. L., I. Huq, A. R. M. A. Alim, S. So, M. Samaspour-Motalebi, and S. Falkow.** 1980. Detection of enterotoxigenic *Escherichia coli* by DNA colony hybridization. J. Infect. Dis. **142**:892–898.
11. **Nishibuchi, M., J. E. Galen, and J. B. Kaper.** 1984. The tdh gene (thermostabile direct hemolysin gene) of *Vibrio parahaemolyticus*. *In* S. Kuwahara and N. F. Pierce (ed.), Advances in research in cholera and related diarrheas, vol. 4. Martinus Nijhoff, The Hague, Netherlands.
12. **Oliver, J. D., R. A. Warner, and D. R. Cleland.** 1983. Distribution of *Vibrio vulnificus* and other lactose-fermenting vibrios in the marine environment. Appl. Environ. Microbiol. **45**:985–998.
13. **Southern, E. M.** 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**:503–517.
14. **Tacket, C. O., F. Brenner, and P. A. Blake.** 1984. Clinical features and an epidemiologic study of *Vibrio vulnificus* infections. J. Infect. Dis. **149**:558–561.
15. **Tison, D. L., J. Greenwood, M. Nishibuchi, and R. J. Seidler.** 1984. Molecular taxonomy of lactose-fermenting Vibrios, p. 217–238. *In* R. Colwell (ed.), Vibrios in the environment. John Wiley & Sons, Inc., New York.
16. **Tison, D. L., M. Nishibuchi, J. D. Greenwood, and R. J. Seidler.** 1982. *Vibrio vulnificus* biogroup 2: new biogroup pathogenic for eels. Appl. Environ. Microbiol. **44**:640–646.
17. **Twedt, R. M.** 1984. Recovery of *Vibrio parahaemolyticus* and related *Vibrios*. *In* Bacteriological analytical manual, 6th ed. Division of Microbiology, Center for Food Safety and Applied Nutrition, U.S. Food and Drug Administration. Association of Official Analytical Chemists, Arlington, Va.
18. **Wright, A. C., J. G. Morris, Jr., D. R. Maneval, Jr., K. Richardson, and J. B. Kaper.** 1985. Cloning of the cytotoxin-hemolysin gene of *Vibrio vulnificus*. Infect. Immun. **50**:922–924.